Photoreactivation of a Cytoplasmic Virus

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Ultraviolet light-inactivated frog virus 3 is efficiently photoreactivated by chick embryo cells. A cellular enzyme is presumably responsible for this repair of viral deoxyribonucleic acid, for the phenomenon is insensitive to an inhibitor of protein synthesis and is not seen in mammalian cells that are known to lack photoreactivating enzyme. Since frog virus 3 is a cytoplasmic virus, functionally significant amounts of photoreactivating enzyme are probably present in the cytoplasm of chick embryo cells.

INTRODUCTION

Lethal ultraviolet damage to the deoxyribonucleic acid (DNA) of many microbes can be repaired during exposure to light of longer wavelengths (13). The chemical basis of this photoreactivation has been described. One of the principal effects of ultraviolet irradiation on DNA is the production of pyrimidine dimers linked by a cyclobutane ring. These dimers are cleaved by the light-dependent photoreactivating enzyme (13).

We have previously reported that lethal ultraviolet damage to pseudorabies or herpes simplex viruses can be repaired by photoreactivation in chick embryo cells (10). The host cell appears to play a critical role in this process since mammalian cell cultures failed to photoreactivate pseudorabies virus (11). Subsequent assays have demonstrated that the photoreactivating enzyme is indeed absent from the cells of placental mammals (3).

In contrast to their efficient photoreactivation of herpesviruses, chick embryo cells cannot repair ultraviolet-damaged vaccinia virus (7, 10). Among the explanations we had considered for this failure was the possibility that the cellular photoreactivating enzyme was confined to the nucleus and thus had no access to cytoplasmic poxvirus DNA. The description by Granoff et al. (9) of another cytoplasmic DNA virus, frog virus 3 (FV-3), offered the opportunity to test this explanation. We report here some characteristics of the photoreactivation of this virus in chick embryo cells.

MATERIALS AND METHODS

Virus and cells. FV-3 [1.1×10^8 plaque-forming units (PFU) per ml], grown in fathead minnow cells, was kindly supplied by Allan Granoff. It was stored at -60°C and used without further passage for all experiments except those requiring labeled virus. FV-3 was

assayed by a plaque method similar to that described for Sindbis virus (12) except that the cultures were stained after 7 days of incubation at 28°C . Primary chick embryo fibroblasts were grown as monolayers in Falcon plastic bottles as described previously (12). BHK-21 cells were obtained from the American Type Culture Collection and grown in Eagle's medium (6) containing 15% fetal calf serum. This line of cells is apparently sensitive to FV-3 (15) since our virus stock had the same plating efficiency in BHK and chick embryo cells and its titer agreed well with that found by Granoff.

Ultraviolet irradiation. Virus stocks were diluted 10^{-3} in phosphate-buffered saline (5) before irradiation to reduce their absorbance at 260 nm to a negligible level. One-milliliter volumes were exposed at room temperature to a 15-w General Electric germicidal lamp at a distance of 25 cm. The irradiated virus was then stored at -60°C after the addition of rabbit serum to a final concentration of 5%.

Photoreactivation. All photoreactivation was carried out in an incubator at 28°C . The light source, a Westinghouse black light, was placed below the cultures, which were supported by a glass shelf. Thus, the photoreactivating light passed through both the glass shelf and the plastic culture flask before reaching the cells. Both the glass and the plastic had adequate transmittance for the light of the wavelengths known to be active in microbial photoreactivation (13). Cultures were irradiated either before or after addition of the agar overlay.

Measurement of viral uncoating. FV-3 with labeled DNA was prepared by exposing infected BHK cells to ^3H -thymidine (1.5 mCi, 1.8 Ci/mmol) during a single step growth. The virus in the cytoplasmic extract was purified by deoxyribonuclease treatment, differential centrifugation, and equilibrium centrifugation on a potassium tartrate gradient as described by Smith and McAuslan (14). The final preparation contained 2.5×10^{-2} counts per min per PFU. A portion of the purified virus was treated with ultraviolet light, and then both irradiated and unirradiated virus were stored at -60°C after the addition of rabbit serum to a final concentration of 5%. To test for uncoating, labeled

virus was allowed to adsorb to monolayer cultures for 1 hr at room temperature. The inoculum was removed for measurement of unadsorbed acid-precipitable radioactivity, and the washed monolayers were incubated with fresh medium at 28 C for 1 hr, at which time sonic extracts of these cells were examined for acid-precipitable and soluble radioactivity before and after treatment with deoxyribonuclease, as previously described (14). Uncoated viral DNA was defined as acid-precipitable radioactivity that was solubilized by deoxyribonuclease. Nearly all of the radioactivity of the original viral preparation was resistant to this enzyme.

RESULTS

Photoreactivation of FV-3. To test for photoreactivation, ultraviolet-treated virus was allowed to adsorb to chick embryo cultures that were then overlaid with agar-medium and incubated at 28 C. After 3 hr, the cultures were exposed to photoreactivating light at 28 C and then placed in a dark incubator for 7 days. Any virus that was photoreactivated would yield an infectious center and ultimately a plaque. Figure 1 shows that chick embryo cells exposed to photoreactivating light efficiently repaired the ultraviolet damage to FV-3. The photoreactivable sector [as defined by Dulbecco (4)] in this and similar experiments was approximately 0.65; that is, 65% of the lesions produced by ultraviolet light were repaired in the infected cell. This value exceeds that reported for most bacteriophages (1).

The black light used as the source of photoreactivating light had one additional unexpected effect on plaque formation by FV-3. Irradiated cultures generally produced larger and more clearly defined plaques. Some sublethal damage by the black light may have resulted in more rapid or more extensive release of virus by the infected cells. This observation cannot explain the observed photoreactivating effect of the black light since the plating efficiency of unirradiated FV-3 was unaffected by exposure to black light (Fig. 1).

We determined the optimal time for exposure to photoreactivating light by using a single virus preparation that had its viable titer reduced by a factor of 500 through exposure to ultraviolet light for 20 sec. Optimal photoreactivation of this preparation yielded a 50-fold increase in the number of plaques. The irradiated virus was allowed to adsorb to cells in the dark for only 30 min. During subsequent 30-min intervals, the infected cultures were exposed to a suboptimal dose of photoreactivating light, sufficient to repair only about one-half of the potentially photoreactivable lesions. Figure 2 shows that fully one-third of the virus could be photoreactivated during the first 30 min after adsorption. The percentage of inactivated virus open to photoreactivation

FIG. 1. Photoreactivation of FV-3 in chick embryo cells. Samples of virus were exposed to various doses of ultraviolet light and then allowed to adsorb to chick embryo cells. The cultures, overlaid with agar, were incubated at 28 C for 2 hr and then some were exposed to black light at a distance of 15 cm for 1 hr. Incubation was then continued in the dark. Dark cultures (●). Cultures treated with photoreactivating light (○).

then increased rapidly to a maximum at 4 hr after infection. We have also plotted (Fig. 2) the data of Smith and McAuslan on the uncoating of FV-3 as measured by susceptibility of labeled virion DNA to deoxyribonuclease (14). The close coincidence in the kinetics of viral uncoating and appearance of photoreactivability suggests that viral DNA becomes accessible to the photoreactivating enzyme as soon as the virion is uncoated. Thereafter, the infected cells were surprisingly stable in their photoreactivable state. Approximately one-third of the viruses could still be photoreactivated after 1 day at 28 C.

Protein synthesis and the establishment of the photoreactivable state. Since we had previously shown that photoreactivation of pseudorabies virus requires protein synthesis after infection (10), it was of interest to see whether the photoreactivation of ultraviolet-treated FV-3 had a similar requirement. Cultures were treated with sufficient cycloheximide (10 μ g/ml) to reduce the incorporation of ³H-leucine by 97%. Exposure to cycloheximide was begun 2 hr before adsorption of the inoculum and continued until the end of the photoreactivation, which was performed in liquid medium. Cycloheximide was then removed by

FIG. 2. Establishment and stability of the photoreactivable state. FV-3 that had been treated with ultraviolet light for 20 sec was allowed to adsorb to chick embryo cultures for 30 min at room temperature. The inoculum was washed out, and the cultures were incubated at 28 C. At intervals, sets of cultures were exposed to the black light at a distance of 15 cm for 30 min and then reincubated in the dark. The increase in titer due to photoreactivation at each interval is plotted as a per cent of the maximum increase which was observed in cultures irradiated between 3.5 and 4 hr after adsorption. The kinetics of uncoating for FV-3 have been calculated from the data of Smith and McAuslan (14).

washing, and the cultures were overlaid with agar and incubated at 28 C. Table 1 shows that the cycloheximide-treated cultures exhibited a high level of photoreactivation that fell just short of the uninhibited control value. This slight reduction in titer is probably not a consequence of the presence of cycloheximide during the infection, for a similar slight decrease was seen in an additional set of cultures treated with cycloheximide before adsorption only (Table 1). Since a slightly lower concentration of cycloheximide completely blocked the photoreactivation of pseudorabies virus (10), the photoreactivation of FV-3 is apparently independent of either cellular or viral protein synthesis.

Attempted photoreactivation in BHK cells. The above experiments with cycloheximide rule out de novo synthesis of the photoreactivating enzyme after infection. Thus, either a preexisting cellular enzyme or an enzyme carried into the cell by the virion itself must be involved. This latter possibility should be considered because several enzymes of nucleic acid and nucleotide metabolism have recently been found to be associated with various virions. For example, Vilagines and McAuslan (16) have found a nucleotide phosphohydrolase in FV-3.

We attempted to photoreactivate free virions of FV-3 that had been exposed to ultraviolet light

TABLE 1. Photoreactivation of ultraviolet light-inactivated^a frog virus 3 (FV-3) in cells treated with cycloheximide

Treatment with cycloheximide ^b (10 µg/ml)	Subsequent treatment	Plaque-forming units/ml ^c
From 2 hr before viral adsorption until the end of exposure to photoreactivating light	Photoreactivated ^d	2.7×10^3
	Dark	6.4×10^1
None	Photoreactivated	4.4×10^3
	Dark	1.1×10^2
From 2 hr before viral adsorption until 1 hr before adsorption	Photoreactivated	3.1×10^3
	Dark	6.6×10^1

^a FV-3 was exposed to ultraviolet light for 20 sec.

^b Cycloheximide was removed by incubating the cultures in four successive lots of fresh medium during 1 hr at room temperature.

^c Averages of triplicate cultures.

^d Cultures in liquid medium were exposed to the black light at a distance of 15 cm for 30 min beginning 2 hr after adsorption of the virus.

for 20 sec. Exposure to three times the maximum flux for photoreactivation of cell-associated virus showed no increase in titer. Since viable virus was the only assay in this experiment, treatment with the detergents generally used to demonstrate virion-associated enzymes was not possible. Disruption of the virion, however, might not be essential for the detection of photoreactivating activity because the photoreactivating enzyme does not require any soluble cofactor or any source of energy other than the light itself (13).

Our attempts to photoreactivate FV-3 virions in this way were uniformly unsuccessful. Thus, it seemed that a preexisting cellular enzyme was the most likely explanation although a virion enzyme activated by the cell or by the process of viral uncoating could not be excluded. The use of BHK cells should distinguish between these alternatives since cells of placental mammals are devoid of photoreactivating activity (3). Because we were uncertain of the timing or the efficiency of possible photoreactivation in BHK cells, we exposed the infected cells to three times the optimal flux for chick cells at various times after adsorption of ultraviolet-treated virus. Table 2 shows that a preparation of ultraviolet-treated virus that was efficiently photoreactivated in chick embryo cells showed no evidence of photoreactivation in BHK cells.

TABLE 2. Attempted photoreactivation of ultraviolet light-treated^a frog virus 3 (FV-3) in BHK cells

Culture	Time of exposure to photo-reactivating light ^b (hr after adsorption of virus)	Plaque-forming units/ml ^c
BHK	Dark	9.1×10^4
BHK	0-1	8.7×10^4
BHK	2-3	9.0×10^4
BHK	7-8	8.3×10^4
Chick embryo	Dark	9.6×10^4
Chick embryo	1.5-2.0	9.2×10^3

^a FV-3 was exposed to ultraviolet light for 20 sec. The titer of unirradiated virus was 7.5×10^4 and 7.3×10^4 PFU/ml in chick embryo and BHK cells, respectively.

^b Cultures were exposed to the black light at a distance of 9 cm.

^c Average of four replicate cultures.

An alternative explanation of these observations would be that the uncoating of ultraviolet-treated FV-3 was defective in BHK cells whereas it proceeded normally in chick embryo cells. To examine this possibility, we determined the effect of prior ultraviolet treatment on the fate of ³H-thymidine-labeled FV-3 virus in both kinds of cells. Cultures of chick embryo and BHK cells with labeled virus adsorbed were incubated for 1 hr. We then prepared cell extracts and determined the fraction of cell-associated viral DNA that was hydrolyzed by deoxyribonuclease. Table 3 shows that ultraviolet treatment had no effect on the uncoating of FV-3 in either cell type. Thus, if photoreactivating enzyme were present in the cytoplasm of BHK cells, the ultraviolet-treated FV-3 should have been repaired.

DISCUSSION

Our results show that the photoreactivating enzyme that repairs lethally ultraviolet-irradiated FV-3 is supplied by the cell. The synthesis of a virus-determined enzyme is excluded by the demonstration that photoreactivation is insensitive to cycloheximide. If the photoreactivating enzyme were an integral part of the virion, the phenomenon should have been observed in mammalian cells, which lack photoreactivating enzyme, and not just in chick embryo cells that are known to contain it.

The repair of ultraviolet-treated viruses that are known to replicate in certain cellular compartments is useful in indicating the subcellular localization of the photoreactivating enzyme. This enzyme can readily be demonstrated in embryonic chicken cells by direct or indirect assay. Certain

TABLE 3. Uncoating of ultraviolet-treated frog virus 3 (FV-3) in BHK and chick embryo cells^a

Sample	Deoxyribonuclease treatment of sonic extracts		Per cent of virus uncoated
	Acid-precipitable counts/min	Acid-soluble counts/min	
FV-3	1,105	25	2
FV-3 adsorbed ^b to BHK cells and incubated 1 hr at 28 C	308	257	45
FV-3 adsorbed ^b to chick embryo cells and incubated 1 hr	271	235	46
FV-3 ultraviolet treated ^c	1,085	14	1
FV-3 ultraviolet treated, adsorbed ^b to BHK cells, and incubated 1 hr	271	259	49
FV-3 ultraviolet treated, adsorbed ^b to chick embryo cells, and incubated 1 hr	259	231	47

^a All values are the averages of two independent experiments done at the same time.

^b Adsorption took place during 1 hr at room temperature. The efficiency of adsorption, by radioactive measurement, varied from 41 to 48%.

^c FV-3 was exposed to ultraviolet light for 20 sec.

enzymes associated with DNA metabolism have been shown to be located in the nucleus. In the only systematic attempt to determine the subcellular localization of the photoreactivating enzyme, Cook and McGrath (2) examined homogenates of embryonic chicken liver and found 80 to 90% of the activity in the nuclear fraction. They were unable to decide whether the small amount of residual non-nuclear enzyme was an artifact of the isolation or represented real cytoplasmic activity.

All methods that depend upon disruption and subsequent fractionation of cells to demonstrate nuclear localization run the risk of releasing enzyme from slightly damaged nuclei. Our experiments, which employ cells that have been infected only an hour earlier by a single lethally irradiated virion, would appear to circumvent this technical problem. FV-3, which is known to replicate in the cytoplasm (8), is very efficiently photoreactivated by chick embryo cells. A comparison with herpes simplex virus-infected chick embryo cells at 31 C (reference 10, Table 1) shows that FV-3 was actually more efficiently photoreactivated by the same light source. Thus, the cytoplasm, at least in the region in which FV-3 replicates, must con-

tain functionally significant amounts of photo-reactivating enzyme.

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